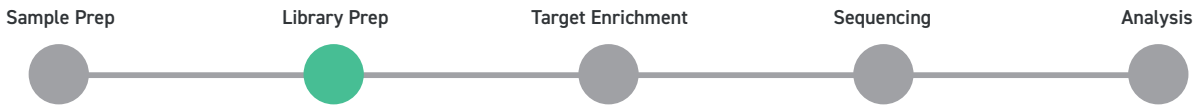
TWIST LIBRARY PREPARATION PROTOCOL

Library Preparation with the Twist UMI Adapter System

For use with the Twist NGS Workflow

This protocol details the steps needed to prepare libraries with unique molecular identifiers (UMIs). It supports a variety of input types including small DNA fragments, such as cell-free DNA (cfDNA), and high molecular weight genomic DNA (gDNA). The workflow generates amplified, indexed libraries with UMI tags for downstream target enrichment and sequencing on Illumina next-generation sequencing (NGS) systems. The products necessary for this workflow include the Twist Library Preparation Kit with Amp Mix, Mechanical Fragmentation and the Twist UMI Adapter System (includes Twist UMI Adapters and Twist Unique Dual Indexed (UDI) Primers). This library preparation protocol is optimized for use with Twist Target Enrichment Kits and should only be performed with reagents specified or their equivalents.



Twist NGS workflow. The complete NGS workflow takes you from sample preparation to NGS sequencing and data analysis. A component of this workflow, the Twist Library Preparation Protocol works in conjunction with the other component protocols.

For Research Use Only. *Not intended for use in diagnostic procedures.*

PROTOCOL COMPONENTS

Please read the product packaging and storage recommendations carefully for each kit and store components as recommended immediately upon arrival.

CATALOG #	NAME	DESCRIPTION	STORAGE
104176: 16 rxn 104177: 96 rxn	Twist Library Preparation Kit with Amp Mix, Mechanical Fragmentation	Reagents for library construction	—
	Twist Library Preparation Kit 1	· 10x ERA Buffer · 5x ERA Enzyme Mix · DNA Ligation Mix · DNA Ligation Buffer · Amplification Primers, ILMN (Tubes 100220, 100583 are not required when used with UMI adapters)	–20°C
	Twist Library Preparation Kit 2	DNA Purification Beads	2–8°C
	Equinox Library Amp Mix	Equinox Library Amp Mix (2x)	–20°C
105040: 16 rxn 105041, 105042, 105043, 105044: 96 rxn	Twist UMI Adapter System - TruSeq Compatible	Twist UMI Adapters and Twist UDI Primers provides UMI labeling and unique dual-indexed combinations with 1 reaction per index pair	–20°C

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MATERIALS SUPPLIED BY USER

The following materials or their equivalent are required to generate libraries using the Twist Library Preparation Kit with Mechanical Fragmentation and Twist UMI Adapter System.

PRODUCT	SUGGESTED SUPPLIER
REAGENTS AND CONSUMABLES	
Ethanol (200 proof)	—
Molecular biology grade water	—
10 mM Tris-HCl ph 8 (optional)	—
Buffer EB (optional)	Qiagen
1.5-ml microcentrifuge tubes	VWR
Thin-walled PCR 0.2-ml strip-tubes	Eppendorf
96-well thermal cycling plates	VWR
1.5-ml compatible magnetic stand	Beckman Coulter
96-well compatible magnetic plate	Alpaqua, Permagen Labware
Qubit dsDNA High Sensitivity Quantitation Assay	Thermo Fisher Scientific
Qubit dsDNA Broad Range Quantitation Assay	Thermo Fisher Scientific
Agilent High Sensitivity DNA Kit (optional)	Agilent Technologies
Agilent DNA 7500 Kit	Agilent Technologies
EQUIPMENT	
Pipettes and tips	—
Vortex mixer	—
Benchtop mini centrifuge for 0.2-ml tubes	—
Thermomixer for 1.5-ml tubes	Eppendorf
Thermal cycler (96 well) with heated lid	—
Fluorometer (Qubit 3.0)	Thermo Fisher Scientific
2100 Bioanalyzer	Agilent Technologies

GENERAL NOTES AND PRECAUTIONS

Wear appropriate protective equipment (lab coat, gloves, and protective glasses or goggles) at all times when performing this protocol.

For best results, read this document before performing the protocol, and follow the instructions provided. Twist cannot guarantee the performance of the Twist Library Preparation Kit with Amp Mix, Mechanical Fragmentation and the Twist UMI Adapter System if modifications are made to the protocol.

This library preparation method may yield more material than needed for target enrichment. Excess product can be stored at –20°C for later use.

Test the compatibility of your thermal cycler and PCR tubes by incubating at 95°C for up to 5 minutes to ensure the PCR tubes do not crack under heat and pressure. Adjust the tightness of the thermal cycler lid and/or use a spacer specific to the thermal cycler model.

GUIDELINES FOR SAMPLES

SMALL MOLECULAR WEIGHT DNA FRAGMENTS

- Mechanical shearing may not be required for small molecular weight DNA samples, for example, cfDNA.
- For optimum performance in downstream target enrichment applications, using samples with minimal high molecular weight DNA background is recommended. The size distribution and purity of the input DNA can be confirmed with an Agilent High Sensitivity DNA Kit before proceeding with end repair and dA-tailing reactions.

HIGH MOLECULAR WEIGHT DNA

- For high molecular weight DNA, such as gDNA, use any desired mechanical shearing method to prepare the fragmented sample.
- For optimum performance in downstream target enrichment applications, analyze the size distribution of the fragmented input DNA with an Agilent High Sensitivity DNA Kit before proceeding with end repair and dA-tailing reactions. Ensure the mode of the fragmentation size distribution is 200–250 bp.

GENERAL

- Use the Thermo Fisher Scientific Qubit dsDNA High Sensitivity Quantitation Assay to accurately quantify DNA input into End Repair, dA-Tailing (Step 1.3, page 10)

⚠ IMPORTANT: For all sample types, accurate input is critical for achieving consistent and optimal results.

- Measuring DNA concentration by absorbance at 260 nm is not recommended.
- Input DNA should be suspended in Molecular Biology Grade Water, 10 mM Tris-HCl pH 8.0, or Buffer EB.
- The recommended DNA input is 30 ng.
- For 30 ng DNA input, a minimum of 20,000x sequencing coverage over the target regions is recommended. Modifying the recommended DNA input will impact the minimal sequencing coverage required for optimal downstream UMI consensus generation, with higher inputs requiring higher sequencing depth.
- Optimization of the following steps in library preparation may be required to achieve optimal performance for a given application.
  - Mass Input of DNA into End Repair, dA-Tailing (Step 1.3, page 10)
  - Amount of Twist UMI Adapter (Step 2.1, page 12)
  - Incubation time for ligation reaction (Step 2.5, page 13)
- For technical support, contact customersupport@twistbioscience.com.

PROTOCOL OVERVIEW

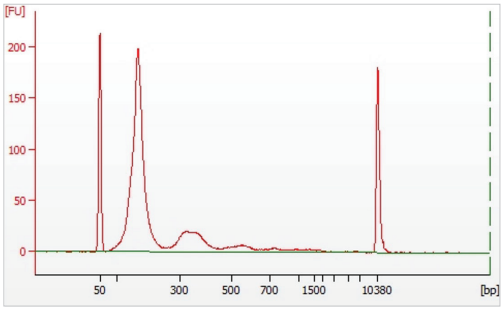
The library preparation with Twist UMI Adapter System protocol supports a variety of input types including intact small molecular weight DNA, such as cell-free DNA (cfDNA), and high molecular weight genomic DNA (gDNA). The protocol generates amplified, indexed libraries with unique molecular identifiers (UMI) for subsequent target enrichment. This protocol allows you to perform DNA library preparation (Steps 1–3) in 3 hours.

Guidance for representative cfDNA and gDNA samples are provided. Other mass inputs and/or sample types may require optimization.

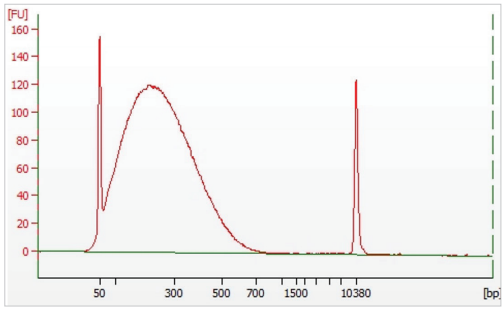
LIBRARY PREPARATION WITH TWIST UMI ADAPTER SYSTEM (30 NG STARTING DNA MATERIAL)	TIME
STEP 1 **Perform End Repair and dA-Tailing** dA-tailed DNA fragments	**1 hour**
STEP 2 **Ligate Twist UMI Adapters and Purify** DNA libraries ready for indexing	**1 hour**
STEP 3 **PCR Amplify Using Twist UDI Primers, Purify, and Perform QC** Amplified indexed libraries	**1 hour**

STEP 1 PERFORM END REPAIR, AND dA-TAILING

Mechanical shearing is not required for cfDNA. Samples with minimal gDNA background are recommended for optimal performance. Size distribution of cfDNA may be checked with an Agilent High Sensitivity DNA Kit before proceeding. cfDNA should have a primary peak at approximately 170 bp and may display multiple secondary peaks with longer fragment lengths.



DNA fragment size distribution of a cfDNA fragment library, as analyzed using an Agilent High Sensitivity DNA Assay



DNA fragment size distribution of a gDNA fragment library immediately after mechanical shearing, analyzed using an Agilent High Sensitivity DNA Assay.

Use any desired mechanical shearing method to fragment gDNA. For optimum performance, analyze the size distribution of the fragmented input gDNA with an Agilent High Sensitivity DNA Kit before proceeding with end repair and dA-tailing reactions. Ensure the mode of the fragment size distribution is 200–250 bp.

Reagents Required

- Input DNA (cfDNA or fragmented gDNA): 30 ng per sample
- Molecular biology grade water
- 10 mM Tris-HCl pH 8 or Buffer EB (optional)
- Qubit dsDNA High Sensitivity Quantitation Assay (or equivalent)
- From the Twist Library Preparation Kit, Mechanical Fragmentation:
  - 5x ERA Enzyme Mix
  - 10x ERA Buffer

Before You Begin

- Thaw 5x ERA Enzyme Mix and mix by flicking the tube with a finger.
- Thaw 10x ERA Buffer on ice, then mix by pulse vortexing for 2 seconds. If the buffer contains a white precipitate, vigorously vortex the buffer until the precipitate dissolves.
- Place molecular biology grade water, 10 mM Tris-HCl pH 8, or Buffer EB on ice.

PREPARE THE THERMAL CYCLER, SAMPLES, AND REAGENTS

1.1 Program the thermal cycler with the following conditions. Set the temperature of the heated lid to 70°C. Start the program to pre-chill the thermal cycler.

	TEMPERATURE	TIME
STEP 1	4°C	HOLD
STEP 2	20°C	30 minutes
STEP 3	65°C	30 minutes
STEP 4	4°C	HOLD

1.2 Use the Qubit dsDNA High Sensitivity Quantitation Assay to determine the concentration of your samples.

⚠ IMPORTANT: For all sample types, accurate input is critical for achieving consistent and optimal results.

1.3 Dilute a total mass of 30 ng DNA with chilled water, 10 mM Tris-HCl pH 8, or Buffer EB to a total volume of 35 µl. Mix well with gentle pipetting.

1.4 Add 35 µl of each diluted DNA sample (30 ng total DNA) into a 0.2-ml thin-walled PCR strip-tube or well of a 96-well thermal cycling plate and place on ice.

NOTE: If a mass input other than 30 ng is desired, dilute the target mass to a volume of 35 µl.

1.5 Pulse-spin to ensure all of the solution is at the bottom of the tube.

PERFORM END REPAIR AND dA-TAILING (ERA)

1.6 Prepare an ERA reaction master mix in a 1.5-ml microcentrifuge tube on ice. Use the volumes listed below. Mix thoroughly by gentle pipetting.

REAGENT	VOLUME PER REACTION*
10x ERA Buffer	5 µl
5x ERA Enzyme Mix	10 µl
Total	15 µl

**Prepare a master mix for multiple reactions.*

1.7 Add 15 µl ERA reaction master mix (from Step 1.6) to each 35 µl DNA sample well or tube, and mix well by gentle pipetting. Cap the tube and keep the reaction on ice.

1.8 Pulse-spin the sample plate or tubes and immediately transfer to the pre-chilled thermal cycler.

1.9 Proceed to steps 2–4 of the thermal cycler program (20°C step of the thermocycler program in Step 1.1 above).

NOTE: While the thermal cycler program is running, prepare the reagents for Step 2 Ligate Twist UMI Adapters and Purify (see Before You Begin).

1.10 When the thermal cycler program is complete and the sample block has returned to 4°C, remove the samples from the block and place them on ice.

PROCEED IMMEDIATELY TO STEP 2: LIGATE TWIST UMI ADAPTERS AND PURIFY

STEP 2 LIGATE TWIST UMI ADAPTERS AND PURIFY

Ligate Twist UMI Adapters to the dA-tailed DNA fragments from Step 1 and purify to generate DNA libraries ready for index introduction through amplification in Step 3.

Reagents Required

- dA-tailed DNA fragments (from Step 1.10)
- Ethanol
- Molecular biology grade water
- 10 mM Tris-HCl pH 8 or Buffer EB (optional, for elution)
- From the Twist Library Preparation Kit 1, Mechanical Fragmentation:
  - DNA Ligation Mix
  - DNA Ligation Buffer
- From the Twist UMI Adapter System:
  - Twist UMI Adapters
- From the Twist Library Preparation Kit 2:
  - DNA Purification Beads

Before You Begin

- Thaw or place on ice:
  - Molecular biology grade water
  - Twist UMI Adapters (tube; utilized for all samples)
- Prepare 1 ml 80% ethanol for each sample (for use in Steps 2 and 3 of the protocol).
- Equilibrate DNA Purification Beads to room temperature for at least 30 minutes (for use in both Steps 2 and 3 of the protocol).
- Program a thermal cycler to incubate the samples at 20°C with the heated lid set to minimum temperature or turned off. Start the program so that the cycler is at 20°C when the samples are prepared.

LIGATE TWIST UMI ADAPTERS

2.1

Add 3 µl Twist UMI Adapters into each sample well or tube containing the dA-tailed DNA fragments from Step 1. Mix gently by pipetting and keep on ice.

2.2

Prepare the ligation master mix in a 1.5-ml microcentrifuge tube on ice as indicated below. Mix well by gentle pipetting.

REAGENT	VOLUME PER REACTION*
Water (chilled)	17 µl
DNA Ligation Buffer	20 µl
DNA Ligation Mix	10 µl
Total	47 µl

**Prepare a master mix for multiple reactions.*

2.3 Add 47 µl of the ligation master mix to the sample from Step 2.1 and mix well by gentle pipetting.

2.4 Seal or cap the tubes and pulse-spin to ensure all solution is at the bottom of the tube.

2.5 Incubate the ligation reaction at 20°C for 15 minutes in the thermal cycler, then move the samples to the bench top. Proceed to the Purify step.

⚠ IMPORTANT: Turn off the heated lid or set to minimum temperature.

NOTE: While the thermal cycler program is running, prepare the reagents for Step 3 (see Step 3: PCR Amplify Using Twist UDI Primers, Purify, and Perform QC).

PURIFY

2.6 Vortex the pre-equilibrated DNA Purification Beads until well mixed.

2.7 Add 80 µl (0.8x) of homogenized DNA Purification Beads to each ligation sample from Step 2.5. Mix well by vortexing.

2.8 Incubate the samples for 5 minutes at room temperature.

2.9 Place the samples on a magnetic plate for 1 minute or until supernatant clears.

2.10 The DNA Purification Beads form a pellet, leaving a clear supernatant. Without removing plate or tubes from the magnetic plate, remove and discard the supernatant.

2.11 Wash the bead pellet by gently adding 200 µl freshly prepared 80% ethanol (do not disturb the pellet). Incubate for 1 minute, then remove and discard the ethanol.

2.12 Repeat the wash once, for a total of two washes, while keeping the samples on the magnetic plate.

2.13 Carefully remove all remaining ethanol with a 10-µl pipet, making sure not to disturb the bead pellet.

NOTE: Before pipetting, the bead pellet may be briefly spun to collect ethanol at the bottom of the plate or tube and returned to the magnetic plate.

2.14 Air-dry the bead pellet on the magnetic plate for 5 minutes or until the bead pellet is dry. Do not overdry the bead pellet.

2.15 Remove the plate or tubes from the magnetic plate and add 17 µl water, 10 mM Tris-HCl pH 8, or Buffer EB to each sample. Mix by pipetting until homogenized.

2.16 Incubate at room temperature for 2 minutes.

2.17 Place the plate or tubes on a magnetic plate and let stand for 3 minutes or until the beads form a pellet.

2.18 Transfer 15 µl of the clear supernatant containing the ligated and indexed libraries to a clean thin-walled PCR 0.2-ml strip-tube or well of a 96-well thermal cycling plate, making sure not to disturb the bead pellet.

PROCEED TO STEP 3: PCR AMPLIFY USING TWIST UDI PRIMERS, PURIFY, AND PERFORM QC

STEP 3 PCR AMPLIFY USING TWIST UDI PRIMERS, PURIFY, AND PERFORM QC

Amplify the adapted DNA libraries with Twist UDI Primers, purify them, and perform quality control (QC) analysis to complete the protocol.

Reagents Required

- Ligated, adapted libraries (from Step 2.18)
- 80% Ethanol (from Step 2)
- Equilibrated DNA Purification Beads (from Step 2)
- Molecular biology grade water
- 10 mM Tris-HCl pH 8 or Buffer EB (optional, for elution)
- Equinox Library Amp Mix (2x)
- From the Twist UMI Adapter System:
  - Twist UDI Primers

⚠ IMPORTANT: Use of Amplification Primers, ILMN tubes 100220, 100583 contained in the Twist Library Preparation Kit 1, Mechanical Fragmentation are not required. Using these primers with the Twist UMI Adapter System will result in a failed PCR amplification.

Before You Begin

Thaw on ice:

- Twist UDI Primers (plate with single use primers)
- Equinox Library Amp Mix (2x)

PREPARE THE THERMAL CYCLER AND PCR MIX

3.1

Program a thermal cycler with the following conditions. Set the temperature of the heated lid to 105°C.

	STEP	TEMPERATURE	TIME	NUMBER CYCLES
1	Initialization	98°C	45 seconds	1
2	Denaturation	98°C	15 seconds	6–10*
	Annealing	60°C	30 seconds	
	Extension	72°C	30 seconds	
3	Final Extension	72°C	1 minute	1
4	Final Hold	4°C	HOLD	–

**6–8 cycles is recommended when starting with 30 ng cfDNA; 8–10 cycles is recommended when starting with 30 ng of high quality gDNA. Further optimization may be needed for other sample types.*

PERFORM PCR

3.2 Add 10 µl of Twist UDI Primer from the provided 96-well plate to each of the DNA libraries from Step 2.18 and mix well by gentle pipetting.

NOTE: For index selection and multiplexing refer to the pooling guidelines in the Appendix.

3.3 Add 25 µl of Equinox Library Amp Mix (2x) to the DNA libraries from Step 3.2 and mix well by gentle pipetting.

3.4 Pulse-spin sample plate or tube and immediately transfer to the thermal cycler. Start the program.

3.5 Remove the sample(s) from the block when the thermal cycler program is complete. Proceed to purification.

PURIFY

3.6 Vortex the pre-equilibrated DNA Purification Beads until mixed.

3.7 Add 50 µl (1x) of homogenized DNA Purification Beads to each ligation sample from Step 3.5. Mix well by vortexing.

3.8 Incubate the samples for 5 minutes at room temperature.

3.9 Place the samples on a magnetic plate for 1 minute.

3.10 The DNA Purification Beads form a pellet, leaving a clear supernatant. Without removing plate or tubes from the magnetic plate, remove and discard the supernatant.

3.11 Wash the bead pellet by gently adding 200 µl freshly prepared 80% ethanol (do not disturb the pellet), incubate for 1 minute, then remove and discard the ethanol.

3.12 Repeat this wash once, for a total of two washes, while keeping the samples on the magnetic plate.

3.13 Carefully remove all remaining ethanol with a 10-µl pipet, making sure not to disturb the bead pellet.

NOTE: Before pipetting, the bead pellet may be briefly spun to collect ethanol at the bottom of the plate or tube and returned to the magnetic plate.

3.14 Air-dry the bead pellet on the magnetic plate for 5 minutes or until the bead pellet is dry. Do not overdry the bead pellet.

3.15 Remove the plate or tubes from the magnetic plate and add 22 µl water 10 mM Tris-HCl pH 8, or Buffer EB to each sample. Mix by pipetting until homogenized.

3.16 Incubate at room temperature for 2 minutes.

3.17 Place the plate or tubes on a magnetic plate and let stand for 3 minutes or until the beads form a pellet.

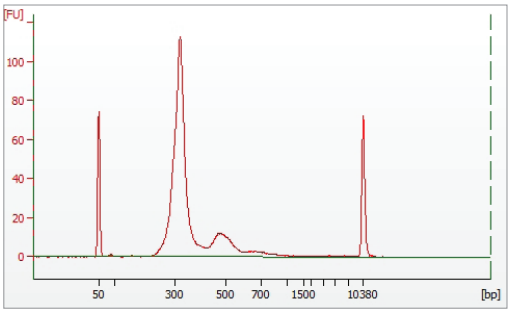
3.18 Transfer 20 µl of the clear supernatant containing the Amplified Indexed Libraries to a clean thin-walled PCR 0.2-ml strip-tube or well of a 96-well thermal cycling plate, making sure not to disturb the bead pellet.

PERFORM QC

3.19 Quantify and validate the size range of each library using the Thermo Fisher Scientific Qubit dsDNA Broad Range Quantitation Assay and Agilent DNA 7500 Assay.

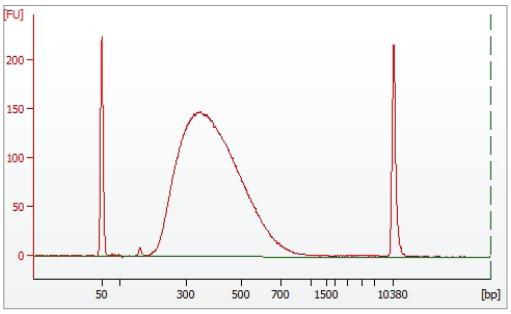
30 ng of cfDNA or high quality fragmented gDNA as input with the recommended number of PCR cycles should result in final concentration values of ≥45 ng/µl. Concentrations below 45 ng/µl may reflect inefficient sample preparation and can result in low library diversity after hybridization.

For cfDNA libraries, a primary library peak should be observed at around 320 bp and a secondary peak should be observed around 480 bp.



Electropherogram generated by an Agilent 7500 DNA analysis of a cfDNA library sample that was prepared as described. Note the prominent peak at ~320 bp and secondary peak at ~480 bp.

For fragmented gDNA libraries, an average fragment length should be 375–425 bp using a range setting of 150–1,000 bp.



Electropherogram generated by an Agilent 7500 DNA analysis of a gDNA library sample that was prepared as described. Note the single prominent peak at ~400 bp.

STOPPING POINT: If not proceeding immediately to a Twist Target Enrichment System, store the amplified indexed libraries at –20°C.

END OF WORKFLOW

APPENDIX

UDI SEQUENCES

For a complete guide of the Twist UDI sequences please refer to PDF document DOC-001129 or Excel file DOC-001130. Both files are available for download at twistbioscience.com/resources.

POOLING GUIDELINES

Twist UDI primers are base balanced for next generation sequencing on a column basis. When pooling unique dual-indexed libraries for 8-plex hybridization, it is recommended that libraries be selected from a single column. Multiple columns may be selected in any desired combination across a single plate or multiple plates for sequencing.

Table 1. Twist UDI primer plate layouts and pooling guidelines.
Twist UMI Adapter System; TruSeq Compatible, 16 Samples (105040)

	1	2	3	4	5	6	7	8	9	10	11	12
A	1	9										
B	2	10										
C	3	11										
D	4	12										
E	5	13										
F	6	14										
G	7	15										
H	8	16										

*PLEASE NOTE: The indexes in the 16 sample plate are not the same in 96 samples, Plate A.

APPENDIX

Twist UMI Adapter System: TruSeq Compatible, 96 Samples, Plate A to D (105041, 105042, 105043, 105044)

Plate A.

	1	2	3	4	5	6	7	8	9	10	11	12
A	1	9	17	25	33	41	49	57	65	73	81	89
B	2	10	18	26	34	42	50	58	66	74	82	90
C	3	11	19	27	35	43	51	59	67	75	83	91
D	4	12	20	28	36	44	52	60	68	76	84	92
E	5	13	21	29	37	45	53	61	69	77	85	93
F	6	14	22	30	38	46	54	62	70	78	86	94
G	7	15	23	31	39	47	55	63	71	79	87	95
H	8	16	24	32	40	48	56	64	72	80	88	96

Plate B.

	1	2	3	4	5	6	7	8	9	10	11	12
A	97	105	113	121	129	137	145	153	161	169	177	185
B	98	106	114	122	130	138	146	154	162	170	178	186
C	99	107	115	123	131	139	147	155	163	171	179	187
D	100	108	116	124	132	140	148	156	164	172	180	188
E	101	109	117	125	133	141	149	157	165	173	181	189
F	102	110	118	126	134	142	150	158	166	174	182	190
G	103	111	119	127	135	143	151	159	167	175	183	191
H	104	112	120	128	136	144	152	160	168	176	184	192

Plate C.

	1	2	3	4	5	6	7	8	9	10	11	12
A	193	201	209	217	225	233	241	249	257	265	273	281
B	194	202	210	218	226	234	242	250	258	266	274	282
C	195	203	211	219	227	235	243	251	259	267	275	283
D	196	204	212	220	228	236	244	252	260	268	276	284
E	197	205	213	221	229	237	245	253	261	269	277	285
F	198	206	214	222	230	238	246	254	262	270	278	286
G	199	207	215	223	231	239	247	255	263	271	279	287
H	200	208	216	224	232	240	248	256	264	272	280	288

Plate D.

	1	2	3	4	5	6	7	8	9	10	11	12
A	289	297	305	313	321	329	337	345	353	361	369	377
B	290	298	306	314	322	330	338	346	354	362	370	378
C	291	299	307	315	323	331	339	347	355	363	371	379
D	292	300	308	316	324	332	340	348	356	364	372	380
E	293	301	309	317	325	333	341	349	357	365	373	381
F	294	302	310	318	326	334	342	350	358	366	374	382
G	295	303	311	319	327	335	343	351	359	367	375	383
H	296	304	312	320	328	336	344	352	360	368	376	384

END OF APPENDIX

LAST REVISED: March 21, 2022

REVISION	DATE	DESCRIPTION
3.0	Mar 21, 2022	• Updated images of representative final libraries.
2.0	Feb 16, 2022	• Updated language for clarity.